Attorney Docket No. 015675.P620

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Patent Application of:	
Robin Kurfurst, et al.	Examiner: Gibbs, Terra C.
Application No. 10/584,982	Art Unit: 1635
Filed: April 2, 2007	Confirmation No.: 2408
For: **OLIGONUCLEOTIDE AND THE USE THEREOF FOR MODULATING AN ISOFORM C BETA-1 PROTEIN-KINASE IN THE FORM OF A SKIN DEPIGMENTATION AGENT**	

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir or Madam:

I, Robin Kurfurst, Ph.D., hereby declare that:

1. I am a citizen of France.

2. I currently reside at Avenue de Verdun, F-45800 St. Jean de Braye.

3. I am currently an employee of Perfume Christian Dior S.A..

4. I am a co-inventor of U.S. Patent Application 10/584,982, the above-identified application ("the Application").

5. I have reviewed and understand International Publication No. WO 95/02069 issued to Bennett et al. ("Bennett") and Journal of Biological Chemistry, 1993, Vol. 268:16:11742-11749 of Park et al. ("Park") cited against the claims of the Application in the Office Action mailed on June 16, 2010.

6. In particular, I understand that in rejecting claims 38, 42-57 and 60-67 in the Office Action as obvious in view of Bennett and Park, the Examiner alleges that Bennett discloses a method of treating conditions associated with PKC beta expression including administering a composition including an oligonucleotide capable of hybridizing with genes or gene products coding for protein kinase C beta-1 (PKC beta-1). The Examiner further alleges that Bennett discloses antisense oligonucleotides targeted specifically to PKC beta 1 only or PKC beta 2 only. The Examiner acknowledges that Bennett fails to disclose that a condition associated with PKC beta expression is depigmentation or hyper-pigmentation. The Examiner alleges that Park teaches that PKC beta isoforms are closely associated with melanogenesis. The Examiner further alleges that the inhibition of one beta isoform over another is obvious in view of the cited prior art references.

7. The claimed composition, however, achieves results which are unexpected and unpredictable in view of the teachings of Bennett and Park. In particular, the instant application surprisingly demonstrates that, contrary to the suggestions of Park, the inhibition of PKC beta 1 only is sufficient to inhibit melanogenesis and thus obtain depigmentation without adversely altering other essential skin functions.

8. The inhibition of PKC beta 1 only, instead of both PKC beta 1 and 2, is highly advantageous. Indeed, as indicated in the article titled *The Use of Antisense Strategy to Modulate Human Melanogenesis* (2007) by Lazou et al, which is a post-published article of which I am a co-author and describes the results relevant to the present Application, it is disclosed that in human skin, PKC beta 1 expression is restricted to melanocytes. See Lazou, page 2, right column, attached herewith as Exhibit A. This ensures that inhibition of PKC beta 1 does not also inhibit other essential functions in the skin.

9. In contrast, PKC beta 2 is also expressed in Langerhans cells. See article titled *Protein Kinase C βII Plays an Essential Role in Dendritic Cell Differentiation and Autoregulates Its Own Expression* (2005) by Cejas et al ("Cejas") attached herewith as Exhibit B. Langerhans cells are a particular type of dendritic cell located in the skin, which play the essential role of immune sentinels in

the skin. Moreover, Cejas et al demonstrates that PKC beta 2 plays a crucial role in the differentiation of Langerhans cells.

10. By following the combined teachings of Bennett and Park, one of ordinary skill in the art would have understood to use antisense oligonucleotides targeting both PKC beta 1 and 2, which would permit depigmentation but would also hamper essential functions of the skin.

11. In particular, while Bennett may suggest oligonucleotides targeting PKC beta-1 only, Bennett suggests the use of such oligonucleotides for the treatment of diseases associated to PKC beta-1 only. Specifically, at multiple occurrences in the description, Bennett highlights the importance of specifically targeting PKC isozymes associated to the disease to be treated. See, for example, page 3, lines 14-20 and page 5, lines 7-9 of Bennett.

12. Bennett then discloses oligonucleotides specific for all known PKC isozymes, including PKC beta. Tables 2 to 4 provide oligonucleotides specific for both PKC beta-1 and PKC beta-2 (Table 2), PKC beta-1 only (Table 3), and PKC beta-2 only (Table 4).

13. While Bennett does not disclose any disease associated to PKC beta-1 and PKC beta-2, PKC beta-1 only, or PKC beta-2 only, it is clear from the global teaching of Bennett that the oligonucleotides of Table 2 should be used for diseases associated to both PKC beta-1 and PKC beta-2, oligonucleotides of Table 3 should be used for diseases associated to PKC beta-1 only, and oligonucleotides of Table 4 should be used for diseases associated to PKC beta-2 only. For diseases associated to both PKC beta-1 and PKC beta-2, a combination of an oligonucleotide of Table 2 with an oligonucleotide of Table 4 may be used alternatively.

14. Park, which only refers to PKC beta without indicating which isoform of PKC beta has been tested, would have been interpreted by one of ordinary skill in the art, at the time the invention was made, as involving both PKC beta-1 and PKC beta-2 in melanogenesis. This is supported by the teachings of the document titled *The Molecular Heterogeneity of Protein Kinase C and Its Implications for Cellular Regulation* (1988) to Nishizuka (submitted in the IDS filed on February 5,

2008)("Nishizukal"). This document is cited in Bennett page 3, lines 14-20, and would thus clearly have been consulted by one of ordinary skill in the art.

15. The legend to Figure 1 of Nishizukal states "[t]he βI and βII subspecies, which seem to be derived from a single messenger RNA by alternative splicing, differ from each other only in ~50 amino acid residues at their carboxy-terminal end regions, V_5, and even in this area they possess a high degree of sequence homology". In view of this statement, one of ordinary skill in the art would have concluded that PKC beta-1 and beta-2 isoforms most probably have about the same functions, and would thus have been incited, based on Park, to inhibit both isoforms for depigmentation applications.

16. Nishizuka further mentions, on page 663, second paragraph of the section entitled "*Individual characteristics*" that PKC beta-1 and PKC beta-2 subspecies have undistinguishable kinetic properties in response to stimulation by the same compound. This would further have motivated one of ordinary skill in the art not to target PKC beta-1 only.

17. Nishizuka also indicates on page 662, right column, that PKC beta-2 is expressed more than PKC beta-1, at least in brain. Furthermore, Table 1 on page 662 indicates that PKC beta-2 is expressed in many tissues and cells, while PKC beta-1 is only expressed in some tissues and cells. This would also have deterred one of ordinary skill in the art to target PKC beta-1 only, since PKC beta-2 has higher probability to be expressed in large quantities in melanocytes.

18. Thus, despite the fact that the prior art would have deterred one of ordinary skill in the art from targeting PKC beta-1 only for depigmentation purposes as claimed, I along with my co-inventors found unexpectedly that the specific targeting of PKC beta-1 is sufficient to inhibit melanogenesis. See, for example, the experiments of Examples 2-4 in which only oligonucleotides targeting PKC beta-1 are used.

19. Accordingly, even if the teachings of Bennett and Park are combined as proposed in the Office Action, one of ordinary skill in the art would not arrive at a method of depigmenting or bleaching human skin, body hair or hair of a head of a subject by applying a composition capable of specifically

hybridising with genes or gene products coding for protein kinase C beta-1 (PKC beta-1) and modifying expression of only PKC beta-1 as claimed.

20. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Respectfully submitted,

Dated: Dec 13, 2010

Robin Kurfurst, Ph.D

EXHIBIT A

THE USE OF ANTISENSE STRATEGY TO MODULATE HUMAN MELANOGENESIS

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Abstract

Background and Objectives: Skin without significant dyschromia is an aesthetic goal of people worldwide. Current options for lightening skin could have significant drawbacks. The antisense strategy may be a viable alternative. The reactions in melanogenesis are catalyzed mainly by tyrosinase, tyrosinase-related protein 1 (TRP-1), and TRP-2. Activation of tyrosinase is associated with phosphorylation by protein kinase C-ßI (PKC-ßI) and formation of a complex between phosphorylated tyrosinase and TRP-1. The aim of this study was to use 2 antisense oligonucleotides to modulate the synthesis of the tyrosinase/TRP-1 complex, PKC-ß, or both by interacting with the targeted mRNA, thus whitening skin by interfering with melanogenesis at the translational level.

Methods/Study Design: In the *in vitro* study, the effect of the antisense oligonucleotides was evaluated by measuring the rate at which dihydroxyphenylalanine (DOPA) oxidase transforms L-DOPA to DOPAchrome in the pathway for melanin biosynthesis. A reduction in the reaction rate compared to the controls corresponded to a decrease in the enzyme activity and, consequently, to a reduction of the formation of melanin pigments. To evaluate the *in vivo* lightening effect of the antisense oligonucleotides, 30 Asian women volunteers with pigmented spots on both hands applied the test product twice daily for 8 weeks. The test product was applied to 2 marked-off areas of the hand: a pigmented spot (to evaluate the effect of the test product on the color of the spot) and a nonpigmented spot area (to evaluate the effect of the test product on normal skin pigmentation). The lightening effect was evaluated by comparing chromametric and mexametric parameters before treatment, after 4 weeks, and after 8 weeks.

Results: *In vitro* DOPA-oxidase activity was inhibited by 13% in melanocytes treated with the antisense sequence for PKC-ßI alone, by 16% with the antisense sequence for TRP-1 alone, and by 36% with the association of 2 sequences. The inhibiting effect with both sequences required the specific sequences with nonreversed polarities. *In vivo* clinical results showed statistically significant whitening in both pigmented spots and nonpigmented spots when the test product was applied twice daily for 8 weeks by up to 30 Asian women.

Conclusions: The association of TRP-1 and PKC-ßI antisense molecules significantly increased the inhibition of tyrosinase activity on human melanocytes. Antisense oligonucleotides are a new generation of active cosmetic ingredients that offer unprecedented specificity, biological stability, and safety in lightening skin. This is the first report of positive results in a cosmetic based on the use of these new active agents.

Introduction

Melanin exists as 4 biopolymer pigments, eumelanin, neuromelanin, pheomelanin, and mixed melanin pigment, which differ from one another in structure, chemical composition, and physical properties.[1] The optical and chemical filtering properties of epidermal melanin protect the skin from UV-induced damage.[2]

Melanin biosynthesis (melanogenesis) occurs in the melanocytes by a series of enzymatic reactions catalyzed by tyrosinase, tyrosinase-related protein 1 (TRP-1), TRP-2, and other melanocyte-specific gene products.[3] Tyrosinase is the rate-limiting enzyme because it catalyzes the 2 initial reactions of melanin biosynthesis: 1) hydroxylation of tyrosine to L-dihydroxyphenylalanine (L-DOPA) and 2) dehydroxylation of L-DOPA to dopaquinone. Many chemical agents that lighten skin act by inhibiting the activity of tyrosinase.[4] TRP-1 and TRP-2 are also important. TRP-1 forms a complex with tyrosinase, thus affecting the activity of tyrosinase,[3,5] while TRP-2 (DOPAchrome tautomerase) protects the carboxylic acids of melanin.[6] Tyrosinase, TRP-1, and TRP-2 are localized in the melanosomal membranes of cells.

Tyrosinase activity in melanocytes is modulated by several factors. Evidence suggests that activation is associated with phosphorylation by protein kinase C-ß (PKC-ß)[7,8] and subsequent formation of a complex between phosphorylated tyrosinase and TRP-1.[6,9,10] PKC-ß expression was reported to be closely associated to melanogenesis[11] and in human skin PKC-ßI is restricted to melanocytes.

For aesthetic and social reasons, women worldwide prefer skin without significant dyschromia, localized or diffuse. To meet this need, a variety of products to whiten skin have been developed, many formulated to reduce melanogenesis

by decreasing tyrosinase activity using a structural analog of tyrosinase, the natural tyrosinase substrate.

A well-known example is hydroquinone (HQ), a skin-lightening agent that inhibits tyrosinase. HQ has been used since the 1950s in over-the-counter skin-lightening products[12] and is found in a variety of plant-derived food products.[13] Although its therapeutic efficacy is well established, its current clinical use is limited to obtaining generalized depigmentation in diffuse vitiligo[14] because of its potential adverse effects.

HQ's toxicity and tendency to irritate skin[12,13,15] has resulted in the European Committee banning its use in cosmetics and limiting its availability to prescriptions. In the US, the FDA announced in 2006 that on the basis of studies showing some evidence of carcinogenesis in rats and mice treated with HQ, it "cannot rule out the potential carcinogenic risk from topically applied hydroquinone in humans." HQ in low concentrations (1%-2%) in skin bleaching creams has also been shown to cause ochronosis. In light of these data, the FDA has proposed that HQ in skin bleaching products be available only by prescription and used only under medical supervision.[16]

Other plant-derived compounds that whiten skin by modulating activity of melanogenic enzymes have been reviewed in detail.[15]

In general, depigmenting agents (eg, kojic acid, arbutin, ferulic acid, and derivatives of dihydroxybenzene, guaiacol, and resorcinol) are unstable, induce cytotoxicity, are irritating to the skin, or require high concentrations to be effective. Hyperpigmentation has been treated with laser devices, cryotherapy, chemical peels, and superficial dermabrasion. The efficacy and safety of laser treatments are limited by laser-induced epidermal trauma and pigmentary alterations.[15]

The disadvantages of these chemical and physical modalities suggest that new skin-lightening options are needed.[17,18]

As a therapeutic approach, blocking gene expression at the DNA or RNA level through nucleic acid inactivation has enormous potential.[19] The advantages of this approach are that it mimics a natural mechanism and uses ingredients that are potentially not cytotoxic because they are present in all living cells, able to interact with a high level of specificity with targets and exhibit reversible actions.[20] For these reasons, antisense oligonucleotides are strong candidates for more specific skin-lightening ingredients.

Antisense oligonucleotides are small and well-defined synthetic single-stranded nucleic acid fragments. Their base sequence (the antisense) can be specifically designed to be complementary to mRNA (the sense) sequence of the target gene of interest. When the antisense sequence binds to the mRNA sequence, hybridization occurs, which prevents that gene information from causing the synthesis of a protein.[20,21] This concept is illustrated in Figure 1.

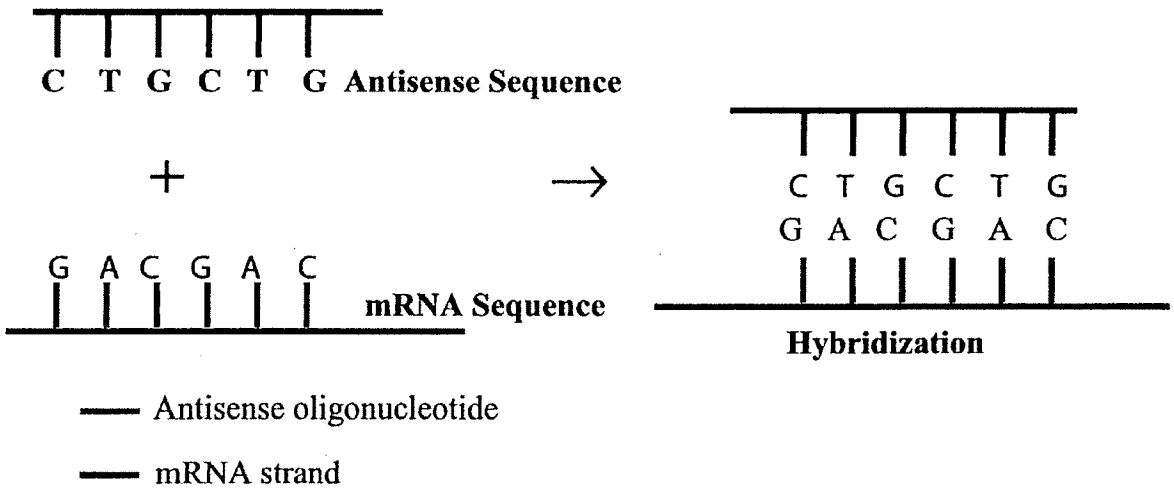
The aim of this study was to use 2 antisense oligonucleotides to modulate the synthesis of the tyrosinase/TRP-1 complex, PKC-ß, or both by interacting with mRNA.

Materials and Methods

In Vitro

The conversion of DOPA to DOPAquinone in melanocytes is a primary reaction in the biosynthesis of melanin. The syntheses of tyrosinase, TRP-1, the tyrosinase/TRP-1 complex, and PKC-ßI are also necessary steps in melanin biosynthesis. The goal of this study was to use two antisense oligonucleotides to modulate the synthesis of the tyrosinase/TRP-1 complex, PKC-ßI, or both in cultured human

Figure 1. Hybridization of sense and antisense sequences prevents the formation of tyrosinase/TRP-1 complex or PKC-ßI. The sense sequence is the natural sequence of base pairs associated with the formation of specific proteins. The antisense sequence of the oligonucleotide is a complementary strand to the sense sequence. In hybridization, adenine (A) pairs only with thymine (T) and guanine (G) pairs only with cytosine (C) respecting the Watson and Crick hybridization scheme.



melanocytes. The 2 oligonucleotides would accomplish this by interacting with mRNA needed to synthesize the tyrosinase/TRP-1 complex and PKC-ßI. The conversion of DOPA to DOPAquinone requires the DOPA oxidase component of tyrosinase. The effect of the antisense oligonucleotides was evaluated by measuring the rate at which DOPA oxidase transforms L-DOPA to DOPAchrome in the pathway for melanin biosynthesis. A reduction in the reaction rate compared to the controls corresponded to a decrease in the enzyme activity and, consequently, to a reduction of the formation of melanin pigments.

The first step was to prepare a pure and confluent culture of normal human melanocytes to which oligonucleotide sequences would be added. Melanocytes were cultured by a standard procedure[18,22] and stored in liquid nitrogen until used. Before treatment with oligonucleotides, frozen cells were thawed at 37°C. The action of the 2 oligonucleotides would be measured by their ability to inhibit the activity of DOPA oxidase in these cultured cells.

The normal human melanocytes were separated from culture by trypsin and then seeded into a 96-well microtiter plate. Treatments were carried out each day for 5 days. For each test, two 96-well plates were processed in parallel, one for DOPA-oxidase activity and the other for cellular viability testing.

Determination of Cellular Viability

Cell viability was monitored to make certain the treatment conditions were not cytotoxic. If they were, a reduction in tyrosinase activity due to cell death and not treatment should be observed in the melanocytes, thus confounding the results.

In parallel with the DOPA-oxidase activity measurements, cellular viability was measured with the XTT test. XTT is a tetrazolium salt, sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6- nitro)benzene-sulfonic acid hydrate.[23] In this colorimetric assay, the mitochondrial dehydrogenases of viable cells reduce tetrazolium XTT salt to water-soluble formazan, which absorbs at 450 nm. The intensity of the colored product is proportional to the number of living cells.

The test was carried out after 5 days of treatment with different oligonucleotides. Normal human melanocytes were seeded in a 96-well microtiter plate (1.10^4 cells/well). Cells were rinsed once with 200 µL PBS per well followed by the addition of 100 µL of XTT solution (0.2 mg/mL in E-199 without phenol red). After the plate was incubated for 3 hours at 37°C in darkness, the absorbance at 450 nm was measured with a spectrophotometer (340 ATTC, STL-LabInstrument, Salzburg, Austria).

DOPA-Oxidase Activity Assay

The assay is based on the 450-nm absorbance of DOPAchrome, the end product formed after the tyrosinase-catalyzed oxidation of tyrosine and DOPA. The absorbance of the colored product measures the activity of the DOPA-oxidase in the tyrosinase-TRP-1 complex.

Before the assay was performed, cells were rinsed with PBS and 50 µL lysis buffer was added to the wells of a microtiter plate. Each well contained cells and lysis buffer. The microplate was agitated at 4°C for 1 hour. Fifty microliters of L-DOPA (10 mM, Sigma) were added to each well. The plate was incubated at 37°C for 1 hour with stirring while the 450-nm absorbance of DOPAchrome was measured (340 ATTC, STL-LabInstrument) at 2-minute intervals. The reaction kinetic measurements were expressed as optical density (OD)/min. For standardization, the values were compared to the values obtained with the technique of the XTT (see next section), which represents the quantity of living cells in each well.

In Vivo

To evaluate the *in vivo* whitening effect of a whitening care test product containing the antisense oligonucleotides 30 Asian women volunteers aged 41 to 63 years (mean 55) with pigmented spots on both hands applied the test product twice daily for 8 weeks under normal conditions of use. The experiment and data analysis were performed at the Institut d'Expertise Clinique in Singapore. The study conformed to the standard operating procedures of the Clinical Research Centre, the general procedures of the Institut D'Expertise Clinique Singapore, the signed protocol, and the general principles of the Good Clinical Practices published by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

The test product was applied to 2 marked-off areas of the hand: a pigmented spot (to evaluate the effect of the test product on the color of the spot) and a nonpigmented spot area (to evaluate the effect of the test product on normal skin pigmentation). The pigmented spot was at least 5 mm in diameter.

The *in vivo* evaluation was based on 1) chromametric measurements of 2 colorimetric parameters and by determination of the individual typological angle and 2) mexametric analysis of the melanin index on the pigmented spots and nonpigmented spot areas. The whitening effect was evaluated by comparing chromametric and mexametric measurements before treatment, after 4 weeks, and after 8 weeks.

Chromametric data were obtained with a Chromameter CR300 (Minolta, Osaka, Japan) device, which measures "L*," a light variable; "b*," a chromaticity coordinate on a blue-yellow axis; and the calculation of the individual typological angle (ITA°) representative of the skin color. Melanin indexes on the pigmented spot and nonpigmented spot area were obtained with a Mexameter MX16 device (Courage & Khazaka Electronic Gmbh, Cologne, Germany).

Homogeneity of variances and normality of distributions of the data were checked by the Levene test and the Komogorov-Smirnov test, respectively. If variances were homogeneous and distributions were normal, differences were analyzed by a paired *t* test (2-tailed); if not, the nonparametric paired Wilcoxon test was used. Significance was set at 5% ($P<.05$).

Results

In Vitro

To determine the concentration of PKC-ßI targeting oligonucleotide necessary to obtain maximum inhibition of DOPA oxidase activity on normal human melanocytes, we treated melanocytes daily for 5 days at 250 and 500 nM concentrations of oligonucleotide. As shown in Figure 2, maximum and statistically significant inhibition occurred at 250 nM. These results were confirmed in a second experiment.

The inhibition of the TRP-1 targeting sequence was evaluated by treating normal human melanocytes daily for 5 days at 250 and 500 nM concentrations of oligonucleotide. As a positive control, melanocytes were treated similarly with kojic acid at 75 µg/mL, the maximum noncytotoxic concentration. As shown in Figure 3, the 500 nM concentration resulted in statistically significant inhibition of DOPA oxidase activity compared to untreated cells. These results were confirmed in a second experiment.

To study the association of both oligonucleotide sequences for a synergistic effect, we treated normal human melanocytes daily for 5 days with the PKC-ßI targeting oligonucleotide (250 nM), TRP-1-targeting oligonucleotide (500 nM), or both sequences in combination. As a positive control, melanocytes were treated similarly with kojic acid at 75 µg/mL. In addition, oligonucleotides corresponding to reversed polarities of TRP-1 (500 nM) and PKC-ßI (250 nM) were also tested. Since these oligonucleotides cannot hybridize on the mRNA sequences of TRP-1 and PKC-ßI, they should not affect DOPA-oxidase activities.

As shown in Figure 4, DOPA-oxidase activity was inhibited by 13% in melanocytes treated with the antisense sequence for PKC-ßI alone, by 16% with the antisense sequence for TRP-1 alone, and by 36% with the association of 2 sequences. In contrast, when melanocytes were treated with oligonucleotides with reversed polarity sequences for TRP-1 and PKC-ßI, DOPA-oxidase activity was not significantly inhibited (Figure 5), showing that the inhibiting effect requires the specific sequences. By taking advantage of the association of both antisense sequences, we achieved a 1.3 times greater inhibition than with either antisense sequence alone.

Figure 2. Study of the concentration of PKC-ßI-targeting oligonucleotide to inhibit DOPA oxidase activity of normal human melanocytes. DOPA-oxidase activity is expressed in OD/min and is compared to the viability obtained with the XTT test. The asterisk (*) indicates statistical significance ($P<.05$). PKC = protein kinase C; OD = optical density; XTT = a tetrazolium salt used to measure cell viability.

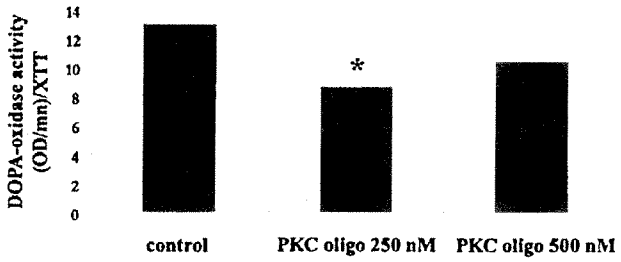


Figure 3. Study of concentration of the TRP-1-targeting oligonucleotide on DOPA-oxidase activity of normal human melanocytes. DOPA-oxidase activity is expressed in OD/min and is compared to the viability obtained with the XTT test. The asterisk (*) indicates statistical significance ($P<.05$). OD = optical density; XTT = a tetrazolium salt used to measure cell viability.

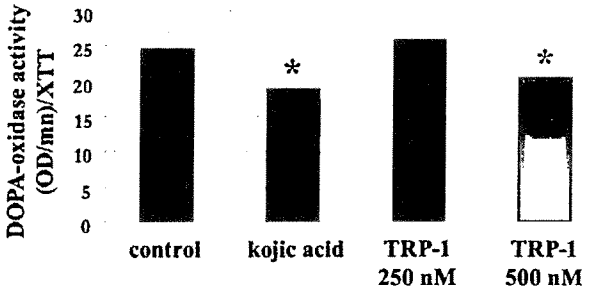


Figure 4. Study of the inhibition of DOPA-oxidase activity by the combination of 2 oligonucleotides targeting the TRP-1 and PKC-ß. DOPA-oxidase activity is expressed in OD/min and is compared to the viability obtained with the XTT test. The asterisk (*) indicates statistical significance ($P<.05$). PKC = protein kinase C; OD = optical density; XTT = a tetrazolium salt used to measure cell viability.

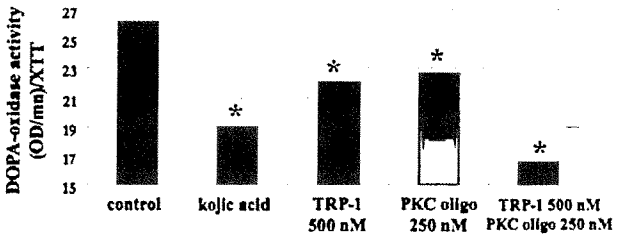
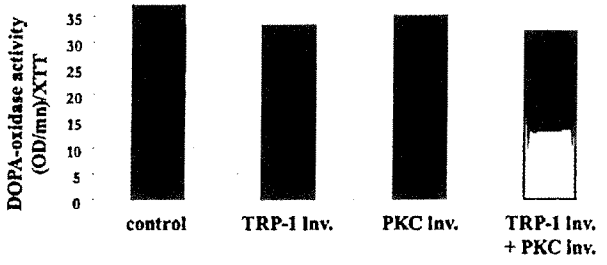


Figure 5. Study of the sequence specificity on DOPA-oxidase activities for TRP-1 and PKC beta-I oligonucleotides using reversed polarity oligonucleotides. DOPA-oxidase activity is expressed in OD/min and is compared to the viability obtained with the XTT test. PKC = protein kinase C; OD = optical density; XTT = a tetrazolium salt used to measure cell viability.



In Vivo

On the area without a pigmented spot, a statistically significant increase in L* was found after 4 weeks (+2%, P=.019) and 8 weeks (+3%, P<.001) of use. A statistically significant increase of the individual typological angle was found after 8 weeks of use (+15%, P<.001). These results indicate that the test product had a lightening effect on the area without a pigmented spot.

On the pigmented spot, a statistically significant increase in L* was found after 4 weeks (+2%, P<.001) and 8 weeks (+4%, P<.001) of use. A statistically significant increase of the individual typological angle (ITA°) was found after 4 weeks (+13%, P=.003) and after 8 weeks (+29%, P<.001) of use (Table 1). These results indicate that the test product had a lightening effect on the pigmented spot.

On the area without a pigmented spot, no statistically significant variation in the melanin index was found after 4 weeks of use, but a statistically significant decrease was found after 8 weeks of use (-0.6%, P<.001), indicating that the test product had a lightening effect on the area without a pigmented spot.

On the pigmented spot, a statistically significant decrease in the melanin index was found after 4 weeks (-0.4%, P=.001) and after 8 weeks (-5%, P<.001) of use, indicating that the test product had a lightening effect on the pigmented spot.

The ITA° of the test product increases significantly (+29%) after 8 weeks of use (Table 1).

Table 1. Results after 4 weeks and 8 weeks of test product.

Parameter	Before Treatment	4 Weeks	8 Weeks	Significance
ITA°	20.2	22.9	26.1	S (P<.01)
Mean %*	—	13%	29%	—

*A variation in percentage compared to before application of test product.
ITA° = individual typological angle.

Discussion

To the authors' knowledge, this is the first report of the use of antisense oligonucleotides as skin-lightening agents for cosmetic purposes. The effect of the antisense oligonucleotides was evaluated by measuring the rate at which DOPA oxidase transforms L-DOPA to DOPAchrome. Quantifying the amount of DOPAchrome produced by this reaction at specific time intervals permitted us to calculate the reaction rate for DOPA oxidase for each oligonucleotide tested and for the controls. A reduction in the reaction rate compared to the controls corresponded to a decrease in the enzyme activity and, consequently, to a reduction of the formation of melanin pigments.[22] DOPA-oxidase activity was inhibited by 13% in melanocytes treated with the antisense sequence for PKC-ßI alone, by 16% with the antisense sequence for TRP-1 alone, and by 36% with the association of 2 sequences. These *in vitro* results were confirmed in an *in vivo* study of 30 Asian women presenting with pigmented spots.

The use of antisense oligonucleotides as therapeutic agents was first reported by Zamecnik and Stephenson in 1978,[24] who synthesized a 13-nucleotide oligonucleotide complement to the terminal sequences of Rous sarcoma virus 35S RNA, which interfered with viral production. A synthetic oligonucleotide can be specifically designed to hybridize a corresponding target mRNA. Its main advantage is specificity, making it possible to selectively modulate gene expression. Other advantages are its biological stability and its efficient uptake and accumulation in cells.[25] The most recent generation of antisense oligonucleotides is well-tolerated and can be safely delivered.[21] Unlike conventional drugs that modulate protein function by binding to proteins, antisense oligonucleotides act upstream by preventing the translation of mRNA into proteins.[25] The first antisense oligonucleotide to be approved by the FDA targets RNA coded by human cytomegalovirus DNA.[19]

Conclusions

The association of TRP-1 and PKC-ßI antisense molecules significantly increased the inhibition of tyrosinase activity on human melanocytes. Antisense oligonucleotides are a new generation of active cosmetic ingredients that offer unprecedented specificity, biological stability, and safety in lightening skin. Until now, a gene silencing technique such as antisense strategy was the only way to act specifically and only on PKC-ßI. The *in vitro* and *in vivo* clinical results of this study are the first reported success in the cosmetic use of these new synthetic agents.

Acknowledgments

We wish to thank Mrs. L. Decup, Mrs. R. Joly, Mrs. E. Duthoit, Mr. F. Wilson, Dr. M. Lemaitre, and Dr. M. Thiry for their kind assistance, fruitful discussions, and support.

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EXHIBIT B

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Protein Kinase C βII Plays an Essential Role in Dendritic Cell Differentiation and Autoregulates Its Own Expression*

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Dendritic cells (DC) arise from a diverse group of hematopoietic progenitors and have marked phenotypic and functional heterogeneity. The signal transduction pathways that regulate the ability of progenitors to undergo DC differentiation, as well as the specific characteristics of the resulting DC, are only beginning to be characterized. We have found previously that activation of protein kinase C (PKC) by cytokines or phorbol esters drives normal human CD34$^+$ hematopoietic progenitors and myeloid leukemic blasts (KG1, K562 cell lines, and primary patient blasts) to differentiate into DC. We now report that PKC activation is also required for cytokine-driven DC differentiation from monocytes. Of the cPKC isoforms, only PKC-βII was consistently activated by DC differentiation-inducing stimuli in normal and leukemic progenitors. Transfection of PKC-βII into the differentiation-resistant KG1a subline restored the ability to undergo DC differentiation in a signal strength-dependent fashion as follows: 1) by development of characteristic morphology; 2) the up-regulation of DC surface markers; 3) the induction of expression of the NFκB family member Rel B; and 4) the potent ability to stimulate allo-T cells. Most unexpectedly, the restoration of PKC-βII signaling in KG1a was not directly due to overexpression of the transfected classical PKC (α, βII, or γ) but rather through induction of endogenous PKC-β gene expression by the transfected classical PKC. The mechanism of this positive autoregulation involves up-regulation of PKC-β promoter activity by constitutive PKC signaling. These findings indicate that the regulation of PKC-βII expression and signaling play critical roles in mediating progenitor to DC differentiation.

Dendritic cells are professional antigen-presenting cells of hematopoietic origin that play a central role in the initiation and regulation of the adaptive immune responses (1–6). Unlike most hematopoietic cells that arise by classical divergent hematopoiesis, human DC can arise from a variety of different immediate precursors. These include CD34$^+$ and CD34$^+$CD86$^+$ hematopoietic progenitor cells (HPC)[1] (7, 8), myeloid progenitors (CFU-M/DC and CFU-DC) (9, 10), peripheral blood monocytes (11–13), and immature neutrophils (14) in what could be considered "convergent" hematopoiesis. In addition to this range of normal progenitors, myeloid leukemia blasts arrested in all stages of differentiation can be driven to undergo DC differentiation (15). On top of this complexity of progenitors, the process of differentiation itself can give rise to DC that are quite different in both their phenotype and function (10), and manipulation of specific signal transduction pathways during differentiation can yield DC with markedly altered function (5, 16). Conversely, exogenous stimuli can inhibit progenitor to DC differentiation in both normal (*i.e.* wound healing) and pathologic (tumors) settings, resulting in suppression of adaptive immune responses (17).

The intracellular signaling pathways that regulate progenitor to DC differentiation are only beginning to be defined. It has been well established that specific membrane receptors (GM-CSF, IL-4, TNF-α, FLT3L, CD40, etc.) can be activated *in vitro* by their exogenous ligands to initiate DC differentiation (7, 11, 18). Downstream of these membrane proximal events, signal transduction involving STAT3 (19, 20), intracellular calcium flux (21–24), specific NFκB family members (25), and Notch family members (26) has been implicated. However, how the various pathways are arrayed in relationship to each other has not been well characterized. We have shown previously that protein kinase C (PKC) activation is necessary and sufficient to drive human CD34$^+$ HPC→DC differentiation (8). It is a likely downstream component of cytokine-mediated differentiation, as PKC inhibitors preferentially block DC differentiation in human CD34$^+$ HPC driven by GM-CSF + TNF-α (27). PKC has been shown to be a downstream component of signaling through the GM-CSF receptor (28) and CD40 (29–31) in other cell types, and these receptors are also centrally involved in DC differentiation. Consistent with a role for PKC in DC lineage commitment, we and others have found that direct activation of PKC signaling by the 2,3-diacylglycerol (DAG) analogs (including phorbol 12-myristate 13-acetate (PMA) and bryostatin-1) in normal CD34$^+$ HPC (8, 32, 33) and myeloid leukemic blasts (27, 34, 35) specifically generates DC in the absence of cell proliferation. Of particular interest is the CD34$^+$CD86$^-$ myeloid leukemia cell line KG1, which we and others have found can be induced to undergo differentiation to fully functional dendritic cells that express DC-specific receptor/chemokines

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[1] The abbreviations used are: HPC, hematopoietic progenitor cells; DC, dendritic cells; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; EMSA, electromobility shift assay; GM-CSF, granulocyte macrophage-colony-stimulating factor; MHC, major histocompatibility complex; GFP, green fluorescent protein; TNF, tumor necrosis factor; IFN, interferon; cPKC, classical PKC; IL, interleukin; DAG, the 2,3-diacylglycerol; bis, bisindolylmaleimide I.

(DC-CK1, DC-STAMP) and have the unique DC ability to cross-present antigens to activate T cells (27, 36). More importantly, a spontaneously arising differentiation-resistant daughter subline KG1a has been derived from KG1, and the two cell lines differ in both PKC activity and isoform expression (37).

PKC comprise a family of 12 serine/threonine protein kinases clustered into three isoform families as follows: classical cPKC (α, βI, βII (alternatively spliced variants of the PKC-β gene), and γ) that are activated by DAG and Ca^{2+}, novel PKCs (δ, ϵ, θ, and η) that are activated by DAG without Ca^{2+}, and atypical (ζ and ι) that do not require DAG and Ca^{2+} (38). It is clear from a wide range of cell types that the different PKCs are not functionally redundant, as specific isoforms modulate specific biological processes. Adapter proteins that bind specific activated PKC isoforms and substrates, such as RACK-1, may further refine the biological effect of PKC activation (39). Relevant to DC differentiation is the finding that PKC-β associates with RACK-1 and the common β chain of the GM-CSF/IL-3/IL-5 receptor, potentially linking cytokine receptor signaling to PKC isoform activation (28). Although a role has been demonstrated for both specific PKC isoforms (40–42) and PKC signal strength (43) in regulating hematopoiesis, involvement of specific PKC isoforms in DC differentiation has not been described previously. We have now extended our previous studies to examine the role and regulation of specific PKC isoforms in DC differentiation.

MATERIALS AND METHODS

Cells and Culture—K562, KG1, and KG1a were obtained from American Type Culture Collection (Manassas, VA) and cultured as described previously (27, 34). $CD34^+$ bone marrow hematopoietic progenitor cells were isolated from organ donor bone marrow under protocols approved by the University of Miami Institutional Review Board, and purified by positive selection as described previously (8). Monocytes were enriched from the mononuclear cells of normal donors by plastic adherence (13). Progenitor cells were differentiated to DC with PMA (10 ng/ml, Sigma) + TNF-α (10 ng/ml, R & D Systems, Minneapolis, MN) for 5–7 days (27, 34) or with GM-CSF (1000 units/ml) and IL-4 (1000 units/ml, both from R & D Systems) for 12 days, and TNF-α (10 ng/ml) for the last 4 days (45). For all differentiation cultures, 50% media changes (with fresh differentiating agents) were performed every 3rd day. Where indicated, cells were pretreated with the PKC inhibitor bisindolylmaleimide I (5 μM). Cell viability was assessed by trypan blue dye exclusion.

KG1a was transfected by electroporation with pcDNA 3.1 (neo vector control), pEGFP-C1 (GFP control), PKC-α-GFP, PKC-βII-GFP, or PKC-γ-GFP (Living Color Vectors; all vectors were from Clontech) and selected for G418 resistance. For PKC-βII-GFP, single cell clones with high or low expression were generated.

Flow Cytometry—Adherent and nonadherent cells were harvested using 3 mM EDTA and stained as reported previously (27) using the following monoclonal antibodies: HLA-ABC and HLA-DR (both from VMRD Inc., Pullman, WA), CD11c, CD14, CD34, CD40, CD80, CD83, and CD86 (all from Immunotech, Westbrook, ME). Appropriate isotype-matched antibodies were used as controls. 10,000 live cells were analyzed on a Coulter XL flow cytometer (Coulter, Hialeah, FL) using the software supplied by the manufacturer.

For MHC class I and II intracellular staining, cells were cultured in media alone or with γ-interferon (250 units/ml, R & D Systems) for 72 h. Cells were then permeabilized using Cytofix/Cytoperm solution (BD Biosciences), as per the manufacturer's instructions, and stained for MHC class I and class II expression as above for flow cytometry.

T Cell Proliferation—Allogeneic T cell proliferation was performed as described previously (27). Progenitors were differentiated as indicated and γ-irradiated (3,000 rads). Normal donor resting allogeneic T cells were purified (>90% $CD3^+$) as described previously (46) and co-cultured at various ratios with differentiated DC for 72 h. 1 μCi/well [*methyl*-^{3}H]thymidine was added for the final 18 h of culture, and incorporation was measured using the Beta Plate scintillation counting system (Wallac Inc., Gaithersburg, MD). Where indicated, T cells alone stimulated with PMA (10 ng/ml) and ionomycin (100 ng/ml) were used as positive controls. The data are presented as the mean of triplicate wells ± S.D.

Confocal Fluorescent Microscopy—$CD34^+$ HPC, monocytes, K562, or KG1 were treated as indicated, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 1% bovine serum albumin, and stained with antibodies against the specific PKC isoforms indicated (anti-PKC-α (clone c-20), PKC-βI (c-16), PKC-βII (c-18), and PKC-γ (c-19), Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then stained with fluorescein isothiocyanate-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) together with Texas Red-X phalloidin and TO Pro-3. Cells were imaged by confocal microscopy (Zeiss LSM-510). Images of typical mid-cell sections are shown.

Western Blot—Western blots were performed as described previously (34). Briefly, protein levels quantitated in cell lysates by using the Micro BCA reagent kit (Pierce). Equal amounts of protein were separated by SDS-PAGE (4% stacking, 10% resolving), electroblotted to nitrocellulose, and probed with antibodies against Rel B (clone c-19), PKC-α (H-7), PKC-βI (E-3), PKC-βII (F-7), and actin (c-2) (all from Santa Cruz Biotechnology) or PKC-γ (Zymed Laboratories Inc.). The proteins were visualized by chemiluminescent detection (ECL, Amersham Biosciences).

Electromobility Shift Assay (EMSA)—EMSAs were done for NFκB family members as described previously (27). Briefly, equal amounts of protein from nuclear lysates were incubated with ^{32}P-labeled primer containing consensus NFκB-binding sites (GAT CCA ACG GCA GGG GAA TTC CCC TCT CCT TA) and separated on 4% polyacrylamide gels. For supershift assays, samples were first incubated with anti-Rel B antibody (Santa Cruz Biotechnology). Samples were visualized by autoradiography.

Northern Blot—Northern blot analysis was performed as described previously (47). Briefly, total RNA was isolated (RNeasy, Qiagen, Valencia, CA) and equalized by serial dilution and ethidium bromide visualization, separated on 1% formaldehyde/agarose gel, transferred to nylon membranes, and hybridized against PKC-βII-specific or glyceraldehyde-3-phosphate dehydrogenase probes.

PKC Kinase Assay—PKC kinase activity levels were determined using the SignaTECT Assay System (Promega), according to manufacturer instructions. Briefly, 7×10^6 cells each of KG1, KG1a, KG1a 3.1 (vector alone), and KG1a E9 (PKC-βII transfected) were homogenized in 25 mM Tris buffer (pH 7.4) containing 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 50 μg/ml phenylmethylsulfonyl fluoride and purified through a DEAE column in a 5-ml final elute volume. A 5-μl aliquot of each sample was incubated in a buffer containing calcium, peptide target, and [α-^{32}P]ATP with and without phospholipids for 5 min at 30 °C, when the reaction was stopped with 7.5 M guanidine-HCl buffer. Each reaction was spotted onto binding paper and quantified by scintillation counting. PKC activities in picomoles of ATP/min were determined as indicated in the kit protocol, normalized to their respective protein content, and expressed relative to the activity of the undiluted (1:1) KG1a cell lysates.

PKC-β Promoter Analysis—The BAC clone RP11-548B6 (Children's Hospital Oakland-BACPAC Resources, Oakland, CA) was digested with SacI and XbaI, and the resulting 1.7-kb DNA fragment containing the PKC-β promoter was cloned into the same corresponding sites in the pBlueScript SK+ vector. The new clone was incubated with NotI and blunted before further digestion with SacI to yield a 1.2-kb fragment (spanning the region from +65 to −1092 relative to the start of transcription) that was cloned into the SacI and SmaI sites of the pGL3-basic vector (Promega, Madison, WI) to yield the reporter construct in forward orientation (pPKC-Forward). The promoter in reverse orientation (pPKC-Reverse) was generated after ligation of the 1.2-kb fragment with the pGL3-basic vector digested previously with KpnI, blunted, and cut again with SacI. The identity and orientation of each clone were confirmed by restriction enzyme digest and DNA sequencing. KG1a-PKC-βII-GFP (E9) cells and KG1a-neo cells were incubated for 24 h at a concentration of 0.25×10^6 and 0.5×10^6 cells/ml, respectively, before electroporation with the Nucleofector system (Amaxa Biosystems, Cologne, Germany) using the T-01 program. Briefly, 10×10^6 were resuspended in 100 μl of reagent V and mixed with 10 μg of the reporter constructs plus 100 ng of the pRL-CMV plasmid (Promega) encoding *Renilla* luciferase to normalize for transfection efficiency. Cells were transfected also with the pDsRed2-C1 plasmid (BD Biosciences) plus pRL-CMV to normalize the *Renilla* luciferase expression levels between both cell lines. Twenty four hours after transfection, cells were harvested, lysed, and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

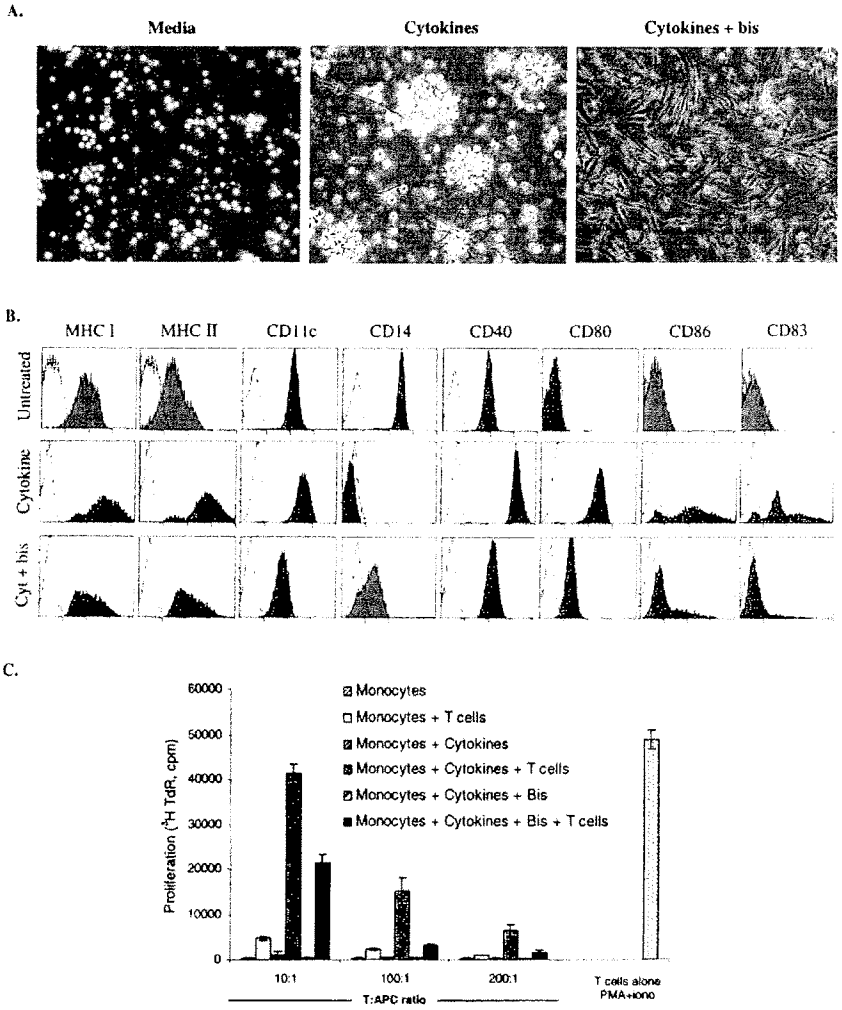

FIG. 1. **PKC inhibitors block cytokine-mediated monocyte to DC differentiation.** *A*, morphology. Monocytes were enriched from normal volunteers and cultured in media alone (*Media*) or differentiated to monocyte-derived DC by culture in cytokines (GM-CSF + IL-4 + TNF-α (*Cytokines*)) + the PKC inhibitor bisindolylmaleimide I (*Cytokines + bis*). Photomicrographs taken at ×200. *B*, immunophenotype. Undifferentiated monocytes and monocyte-derived DC generated above were stained by the indicated antibodies and analyzed by flow cytometry. Open histograms are the isotype controls. *C*, induction of allogeneic T cell proliferation. Undifferentiated monocytes or monocyte-derived DC were generated as above in the absence (*Monocytes + Cytokines*) or presence (*Monocytes + cytokines + Bis*) of bisindolylmaleimide, irradiated, and cultured in the absence or presence (*+T cells*) of 2×10^5 purified normal allogeneic T cells at the indicated ratios. As a positive control, 2×10^5 T cells from the same donor were cultured alone with PMA + ionomycin. [^{3}H]Thymidine incorporation was measured for the last 18 h of culture. Data are presented as mean proliferation ± S.D. of triplicate wells. Data are representative of two independent experiments.

RESULTS

Blockade of PKC Activation Inhibits Cytokine-mediated Monocyte→DC Differentiation—We have shown previously that that the PKC-specific inhibitor bisindolylmaleimide I (bis) could inhibit cytokine-driven DC differentiation in primary CD34$^+$ HPC (27). To assess whether this was unique to CD34$^+$ HPC or represented a common role for PKC in downstream cytokine signal transduction, we first examined whether bisindolylmaleimide I could inhibit cytokine-mediated generation of dendritic cells from primary human monocytes *in vitro*. We evaluated the effect on three DC characteristics as follows: morphology, immunophenotype, and the ability to activate allogeneic T cells. Morphologically, undifferentiated monocytes were loosely adherent round cells (Fig. 1A, *left panel*), whereas monocytes cultured with GM-CSF + IL-4 + TNF-α differentiated into clusters of DC (48) (*center panel*). Inhibition with bis resulted in the loss of these clusters (Fig. 1A, *right panel*), with a predominance of adherent spindle-shaped cells. Bis also blocked cytokine-mediated up-regulation of characteristic DC markers (MHC I and II, CD11c, CD40, CD80, CD86, and CD83) while preventing the complete down-regulation of the monocyte marker CD14 (Fig. 1*B*). The immunophenotype of these bis-treated monocytes appeared to be that of activated monocytes. Functionally, inhibition of PKC activation generated cells that were significantly less able to induce allogeneic T cell proliferation at all T cell:APC ratios tested (especially at the higher ratios) but were more immunostimulatory than undifferentiated monocytes (Fig. 1*C*). The latter observation would also be consistent with the bis-treated cells being activated monocytes. Together, these data suggest that PKC activation is also a downstream component of cytokine receptor-mediated signaling of DC differentiation from monocytes.

PKC-βII Is Specifically Activated by DC Differentiation Signals—To determine which PKC isoform(s) were being activated

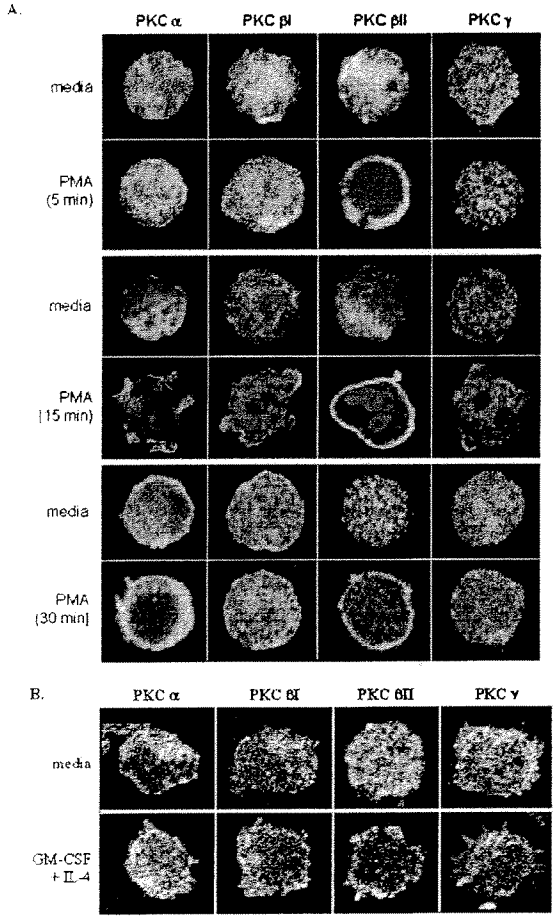

FIG. 2. **PKC-βII is specifically activated by DC differentiation signals.** *A*, PMA-induced differentiation. Purified normal human $CD34^+$ bone marrow hematopoietic progenitor cells (*$CD34^+$ HPC*), K562, and KG1 were stimulated with PMA for the times indicated, stained for the indicated PKC isoform (*green*), actin (*red*), and the nucleus (*blue*), and imaged by confocal microscopy as detailed under "Materials and Methods." Data are representative of four independent experiments. *B*, cytokine-induced differentiation. Primary human monocytes were undifferentiated (*media*) or cultured in GM-CSF + IL-4 for 24 h and imaged as above. Data are representative of two independent experiments.

during the initiation of DC differentiation, we took advantage of the fact that PKC activation results in translocation of the enzyme from the cytoplasm to the plasma membrane (49) and can be directly visualized by confocal microscopy. We focused on the DAG/Ca^{2+}-dependent cPKC family (α, βI, βII, and γ), as we have found evidence for involvement of calcium in PMA-mediated DC differentiation (27, 34). In three different progenitors that we have shown previously differentiated to DC in response to PMA (primary $CD34^+$ HPC, KG1, and K562), all four PKC isoforms are cytoplasmic when cells were unstimulated (*media*, Fig. 2*A*). Within 5–30 min of PMA stimulation, only the PKC-βII isoform translocated to the plasma membrane in all three cell types. Translocation of PKC-βII was also seen when primary monocytes were differentiated with GM-CSF + IL-4 for 24 h (Fig. 2*B*). This translocation took somewhat longer to detect compared with PMA treatment but is consistent with the slower kinetics of DC differentiation we see with cytokines *versus* PKC agonists. These findings indicate there is immediate/early activation of PKC during DC differentiation and that PKC-βII may specifically mediate this lineage commitment.

Restoration of DC Differentiation in KG1a Transfected with PKC Isoforms—We and others have shown that the human $CD34^+CD86^+CD14^-$ myeloid leukemia cell line KG1, which has the same phenotype as primary $CD34^+CD86^+$ human DC progenitors (50), similarly undergoes differentiation in response to cytokines or PMA to dendritic cells that have DC-specific molecular expression (*e.g.* DC-CK1 and DC-STAMP) and function (*e.g.* cross-presentation of antigen) (27, 35, 36, 51–55). KG1a is a less differentiated cell line spontaneously derived from KG1 and does not differentiate in response to phorbol esters (27, 56, 57). KG1a has less overall PKC activity and lower expression of βI and βII compared with KG1 (27, 37). Given our demonstration that PKC-βII activation occurs during KG1 differentiation, we next asked if transfection of specific PKC isoforms into KG1a could restore the ability of these cells to differentiate to DC. As seen in Fig. 3*A*, KG1a was stably transfected with specific functional PKC isoform-GFP fusion constructs (58). Analysis of bulk cultures of the KG1a-PKC-βII transfectants revealed both high and low expressing clones. Single cell cloning resulted in isolation of each type of cell. Confocal imaging for GFP demonstrated cytoplasmic localization for PKC-α-GFP, PKC-βII-GFP, and PKC-γ-GFP in the unstimulated KG1a transfectants (Fig. 3*B*). PKC-α-GFP also appears as punctate cytosolic accumulations in unstimulated cells, which have been reported previously in NIH-3T3 cells (59). The cytosolic localization in unstimulated cells demonstrated that the majority of the transfected enzyme was not constitutively activated. Following PMA treatment, only PKC-βII-GFP underwent significant translocation to the plasma membrane. In addition to the plasma membrane, PKC-βII-GFP also translocated elongated subcellular structures that appeared to be contiguous with the plasma membrane on stacked views (Fig. 3*B*, *arrow*). These results are consistent with our findings with the endogenous PKC isoforms and indicate that the different PKC-GFP isoforms transfected into KG1a behave in a similar fashion to the endogenous PKC isoforms in KG1 (as well as in primary $CD34^+$ HPC and monocytes).

We next assessed the ability of PKC-transfected KG1a to undergo DC lineage commitment in response to PMA. We have shown previously in KG1 and $CD34^+$ HPC that this is characterized by development of typical DC morphology with extended branching dendrites, decreased proliferation and increased cell death, acquisition of DC surface markers (MHC I, II, CD40, CD80, CD86, and CD83), up-regulation of the NFκB family member Rel B, and the ability to induce allogeneic T cell proliferation.

Morphology—Recent studies have demonstrated the importance of the extended dendrite morphology in the ability DC to activated T cells (60). We have found previously that in contrast to the clusters of cells with short dendrites induced by cytokine combinations, PMA-induced DC differentiation from primary $CD34^+$ HPC or KG1 results in more tightly adherent individual cells with longer processes (either spindle shaped or branching) (27) as seen in Fig. 4*A* for KG1 (8). Both unstimulated (media) vector control KG1a and PKC-transfected cells were morphologically round and loosely adherent (Fig. 4*A*). Treatment with PMA had little effect on vector control KG1a morphology. However, similar to the parental KG1 line, PMA stimulation induced development of tightly adherent cells with extended cytoplasmic processes in the PKC-βII-GFP transfected cells. Although the KG1a PKC-α-GFP and PKC-γ-GFP cells also became adherent with some spindle-shaped cells, many of the cells were adherent round cells with very large vacuoles that is not a typical DC morphology.

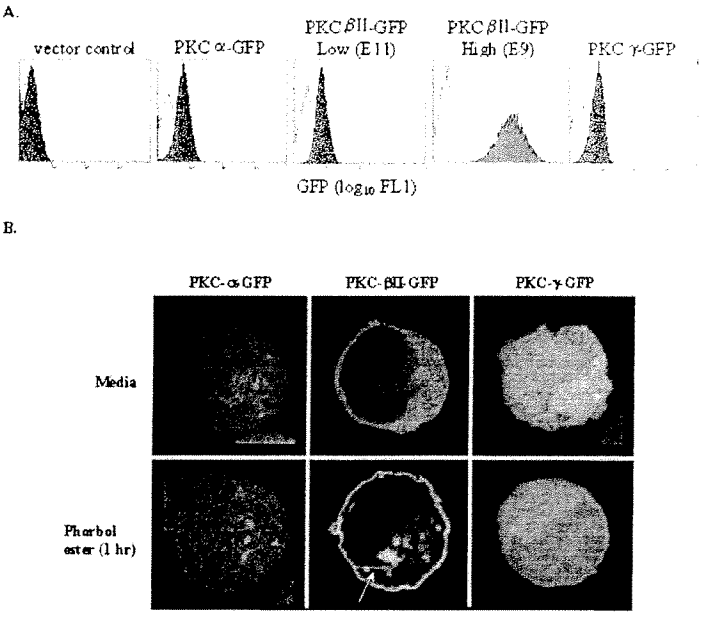

FIG. 3. **KG1a transfection with PKC-GFP.** *A*, flow cytometry. Bulk populations of KG1a stably transfected with neomycin (*vector control*), PKC-α-GFP, PKC-γ-GFP, and single cell clones transfected with PKC-βII-GFP (*E9* and *E11*) were analyzed for GFP expression by flow cytometry. *B*, confocal microscopy. The indicated KG1a-PKC-GFP transfectants were left unstimulated (*media*) or stimulated with PMA (*phorbol ester*) and imaged by confocal microscopy for the GFP tag (*green*). *Arrow* points to a PKC-βII-GFP-positive subcellular structure. Data are representative of three independent experiments

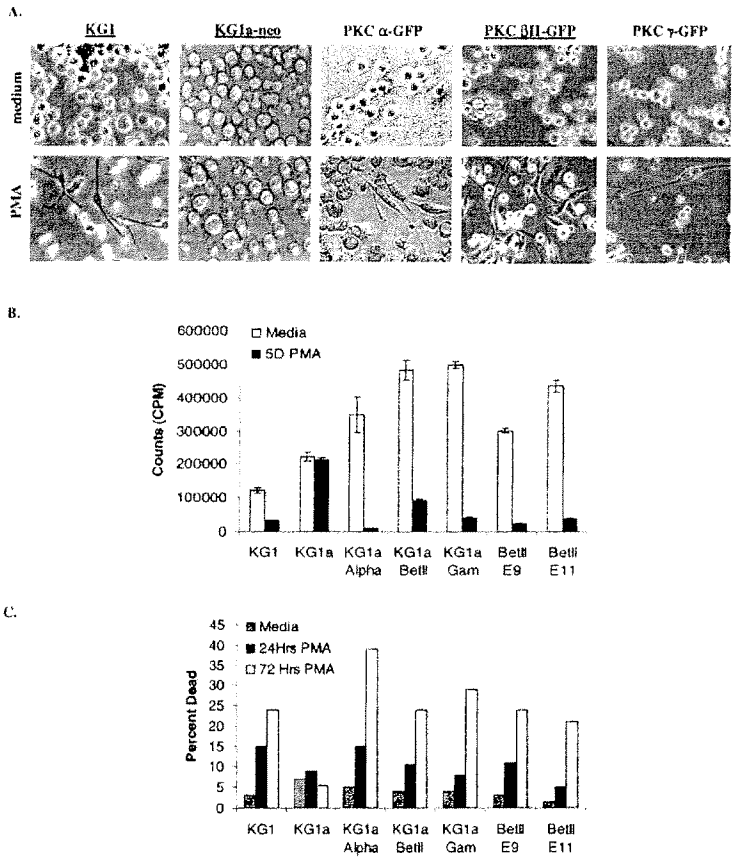

FIG. 4. **PKC transfection restores morphologic changes, proliferation arrest, and cell death associated with PMA-induced DC differentiation.** *A*, morphology. KG1a transfectants were left unstimulated (*medium*) or stimulated with PMA for 5 days (*PMA*). Photomicrographs were taken at ×400. *B*, proliferation. 5×10^5 of the indicated cells were cultured in media or PMA for 5 days, and [^{3}H] thymidine incorporation was measured for the last 18 h of culture. KG1a-PKC-α-GFP (*KG1a-Alpha*), KG1a-PKC-βII-GFP (*KG1a-BetII*), and KG1a-PKC-γ-GFP (*KG1a-Gam*) are bulk transfectant cultures, whereas KG1a-PKC-βII-GFP clones E9 (*BetII E9*) and E11 (*BetII E11*) are single cell clones. Data are presented as mean proliferation ± S.D. of triplicate wells. Data are representative of three independent experiments. *C*, cell death. Cell viability was measured by trypan blue exclusion in the conditions indicated. Data are representative of two independent experiments.

Differentiation-induced Cell Proliferation Arrest and Death—We have found previously that PMA-induced DC differentiation is associated with arrest of cell proliferation and induction of cell death (20–50%) in all the progenitors we have examined. Compared with KG1, the proliferation of KG1a was largely unaffected by PMA treatment (Fig. 4*B*). Although the KG1a-PKC transfectants have a higher basal rate of proliferation than either KG1 or KG1a, PKC-GFP transfection restored the ability of PMA to inhibit cell proliferation down to levels equivalent to PMA-treated KG1. Similarly, PMA induced

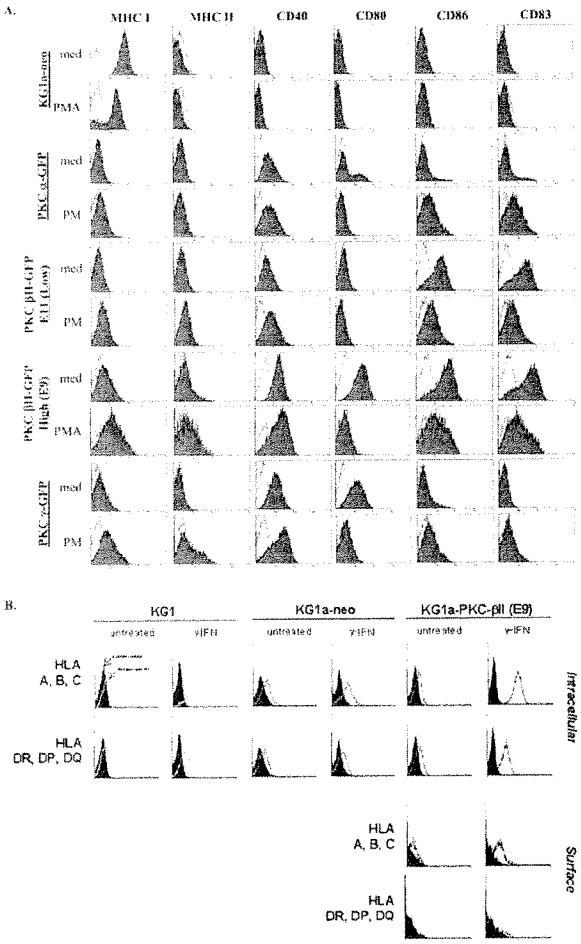

FIG. 5. **KG1a immunophenotype.** *A*, surface staining. KG1a transfectants were left unstimulated (media (*med*)) or stimulated with PMA (*PMA*), stained for the indicated surface markers, and analyzed by fluorescence-activated cell sorter. Isotype controls are the *open histograms*. The results are representative of three independent experiments. *B*, MHC class I and II expression. KG1, KG1a-neo, and KG1a-PKC-βII-GFP clone E9 were left untreated or stimulated with γ-interferon and analyzed for intracellular and surface MHC class I and II staining by flow cytometry. Isotype controls are the *filled histograms*. Data are representative of two independent experiments.

progressively more cell death over time in KG1 but had no effect on KG1a (Fig. 4*C*). However, transfection of either PKC-α-GFP, PKC-βII-GFP, or PKC-γ-GFP restored the ability of PMA to induce cell death in the transfected KG1a to levels equal to or greater than KG1.

Surface Antigen Immunophenotype—KG1 expressed high levels of MHC I and II and low levels of CD86 in media alone and up-regulated CD80 and CD83 upon PMA stimulation (27). In comparison, unstimulated vector control KG1a in media expressed only MHC I, and PMA treatment down-regulated this expression without inducing other DC markers (Fig. 5*A*). Most unexpectedly, all three PKC isoform-transfected KG1a had down-regulated MHC I and up-regulated CD40 and CD86 in media alone. Conversely, unlike KG1 none of the unstimulated transfectants expressed surface MHC II, although it could be detected intracellularly (below). Constitutive expression was also seen for CD80 except for the low expressing βII clone E11 and CD83 (except for PKC-γ-GFP). These findings suggest that some PKC signaling is occurring in the transfected KG1a in the absence of exogenously added PMA. Stimulation with PMA results in variable up-regulation of MHC I in all the transfectants, MHC II (PKC-γ-GFP, PKC-βII-GFP (slight)), CD86 (PKC-α-GFP, PKC-γ-GFP), and CD83 (PKC-α-GFP). Conversely, PMA treatment down-regulated CD80, CD86, and CD83 in transfectants that had high level constitutive expression of these markers. We have found previously that PMA treatment can down-regulate constitutive expression of MHC I, MHC II, CD80, and CD86 in DC progenitors (27, 34). This may represent a complex relationship between PKC signal strength and expression of these markers, which is also suggested by the immunophenotypic differences between the PKC-βII high (E9) and low (E11) expressing clones.

To determine why there was little MHC I and II surface expression in the KG1a-PKC transfectants, we examined whether they were present intracellularly and could be induced by γ-interferon stimulation. As seen in Fig. 5*B* (*upper panels*), KG1, KG1a-neo, and KG1a-PKC-βII (E9) cells all had low constitutive expression of intracellular MHC I and II. γ-IFN treatment induced expression of both in KG1, whereas there was less (MHC I) to no effect (MHC II) in KG1a-neo cells. In contrast to the control neo cells, KG1a-PKC-βII (E9) regained the ability to up-regulate both intracellular MHC I and II in response to γ-IFN. Although this up-regulation resulted in increased MHC I surface expression, there was virtually no increase in MHC II surface expression (Fig. 5*B*, *lower panels*). We have also found that that the addition of TNF-α (as a maturation factor) to γ-IFN treatment does not alter MHC I or II expression compared with γ-IFN alone, nor does the addition of γ-IFN during PMA-induced differentiation alter MHC I or II expression compared with γ-IFN alone (not shown). These findings suggest that although γ-IFN-inducible MHC II gene expression is reconstituted by PKC-βII transfection, MHC II transport to the cell surface remains impaired. We have also found that compared with KG1, there is low expression of the chaperone protein Erp57 (which is involved in peptide loading onto MHC I complexes) in KG1a that is not increased in KG1a-PKC-βII cells (data not shown), which may explain the low basal levels of MHC I surface expression in these cells.

Rel B Expression—Immediate/early up-regulation of expression of the NFκB family member Rel B is an essential event for both DC differentiation (61) and function (62). It is the inducible nature of Rel B that is important, as it has been shown that constitutive expression of Rel family members will actually inhibit DC differentiation (63). Although KG1 have low levels of Rel B that was rapidly induced following PKC activation, KG1a had higher levels of constitutive expression that was not inducible (Fig. 6*A*). Transfection of PKC-α, -βII, or -γ down-regulated Rel B expression in unstimulated cells, which was then inducible by PMA stimulation. The restoration of a KG1-like pattern of inducible Rel B protein expression was also reflected in the regulation of nuclear Rel B capable of binding NFκB DNA motifs as detected by EMSA (Fig. 6*B*). Most surprisingly, the total NFκB DNA binding activity was down-regulated in the unstimulated KG1a-PKC-βII and was inducible by PMA, suggesting a common mechanism of NFκB regulation that is not specific for Rel B.

T Cell Proliferation—Functionally, restoration of the ability of KG1a to undergo DC differentiation by transfected PKC isoforms should be reflected in the re-acquisition of T cell stimulatory capacity. As seen in Fig. 7*A* (*left panel*), undifferentiated KG1, KG1a, or KG1a transfectants did not induce allogeneic T cell proliferation. The fact that the undifferentiated PKC-βII E9 clone expresses MHC I, MHC II (slight), CD40, CD80, and CD86 and yet still did not elicit T cell prolif-

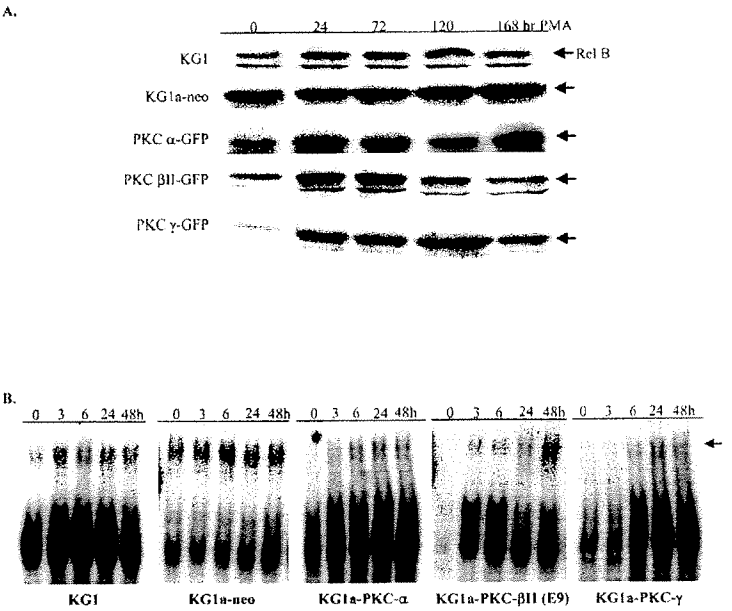

FIG. 6. **Rel B expression.** *A*, protein. KG1 and the KG1a transfectants were stimulated with PMA as indicated; protein lysates were isolated, and equal amounts were analyzed for Rel B by Western blots. *Arrow* points to the specific band for Rel B. Results are representative of three independent experiments. *B*, EMSA. KG1 and the KG1a transfectants were stimulated with PMA as indicated; protein lysates were isolated, and equal amounts were analyzed for binding to labeled DNA probes containing the consensus NFκB-binding site. *Arrow* points to the complexes supershifted by an anti-Rel B antibody. Data are representative of two independent experiments.

eration suggests factors other than antigen presentation receptors and co-stimulatory ligands are necessary to elicit T cell activation. KG1 differentiated with PMA, and more so with addition of the maturation signal TNF-α, can stimulate T cell proliferation. By comparison, PMA + TNF-α-treated KG1a, KG1a vector control, and KG1a-GFP do not acquire allostimulatory capacity. These controls also demonstrate that PMA carryover from the differentiation cultures does not account for the T cell proliferation seen in the co-cultures. In contrast, PMA-induced differentiation of all three PKC-transfected KG1a resulted in the ability to drive significant T cell proliferation. This was significantly greater than undifferentiated monocytes (Fig. 7*A*, *right panel*, using the same donor T cells), and was equivalent to monocyte-derived DC.

We next assessed whether the degree of PKC-βII expression affects the ability to induce allo-T cell proliferation. As seen in Fig. 7*B*, the high expressing βII E9 clone consistently induced greater T cell activation than the low expressing E11 clone, implying that the strength of the PKC signal may affect the immunogenicity of the resulting DC. Altogether, these data demonstrate that transfection with PKC-α-GFP, PKC-βII-GFP, or PKC-γ-GFP can reconstitute the ability of KG1a to undergo DC lineage commitment as seen for KG1.

PKC Induction of Endogenous PKC Isoform Expression—Because we have consistently observed activation of PKC-βII and not other cPKC isoforms during DC differentiation, we were surprised by the ability of the α and γ isoforms to also restore aspects of DC differentiation in the KG1a cells. We sought to understand the underlying mechanism by first comparing the relative levels of endogenous PKC *versus* PKC-GFP expression in the transfected KG1a. Because of the GFP tag, we can distinguish transfected PKC from endogenous PKC by molecular weight on Western blots. Most unexpectedly, we found relatively low levels of transfected PKC-GFP protein expression (Fig. 8*A*). Except for the high expressing PKC-βII-GFP clone E9, the transfected PKC-GFP proteins were only detectable upon long exposures. Remarkable, however, was the up-regulation of all the endogenous cPKCs in each of the KG1a transfectants. This is most marked for PKC-βII and demonstrates that although untransfected KG1a did not express much PKC-βII, the ability to up-regulate this expression was still intact. These findings suggested that the transfected PKC-GFPs were not up-regulating PKC activity by their own overexpression but rather by inducing endogenous PKC expression. This would also explain why PKC-α or -γ transfection can restore the ability of KG1a to differentiate to DC, namely via up-regulation of endogenous PKC-βII.

How do the transfected PKC-GFPs induce endogenous PKC-βII expression? Because PKC-βI and -βII are alternatively spliced variants from the PKC-β gene that can be generated in a regulated fashion (64), we first examined if increased PKC-βII expression was because of a switch of splicing from βI to βII. KG1a expressed very little PKC-βI to begin with (Fig. 8*B*), making it unlikely that changes in splicing account for the increased βII expression. The fact that both PKC-βI and -βII protein were induced in the PKC-transfected KG1a suggests that expression of the PKC-β gene is up-regulated. Consistent with this, PKC-β mRNA expression was significantly up-regulated in the KG1a transfectants (Fig. 8*C*). Because PKC enzymes are degraded following activation (65), we determine whether the induced endogenous PKC-βII could be activated by following protein levels during PMA treatment. As seen in Fig. 8*D*, PKC-βII was degraded during PMA stimulation in KG1, KG1a, and all the PKC-transfected KG1a. This indicates that the induced endogenous PKC-βII can be activated and is likely to be signaling. Together, our data indicate that the transfected PKC-GFP are up-regulating endogenous PKC isoform gene expression (in particular PKC-βII), and this confers on KG1a the ability to undergo DC lineage commitment. These findings also suggest that regulation of PKC-βII expression may in turn regulate the ability of progenitors to undergo DC differentiation.

Autoregulation of PKC Promoter Activity—The ability of the transfected PKC-GFP to up-regulate endogenous PKC-βII mRNA expression suggested that this is happening at the transcriptional or post-transcriptional (*e.g.* mRNA stabilization) level. Previous studies have demonstrated a phorbol ester-inducible site in the PKC-β promoter (66, 67), suggesting that PKC activity may positively autoregulate its own gene expression. The relationship of the enzyme activation constant, K_{act} = (active enzyme)/(inactive enzyme), predicts that under steady state conditions, an increase in the total concentration of PKC enzyme (by transfection or induction of endogenous expression) will result in an increase in the number of activated

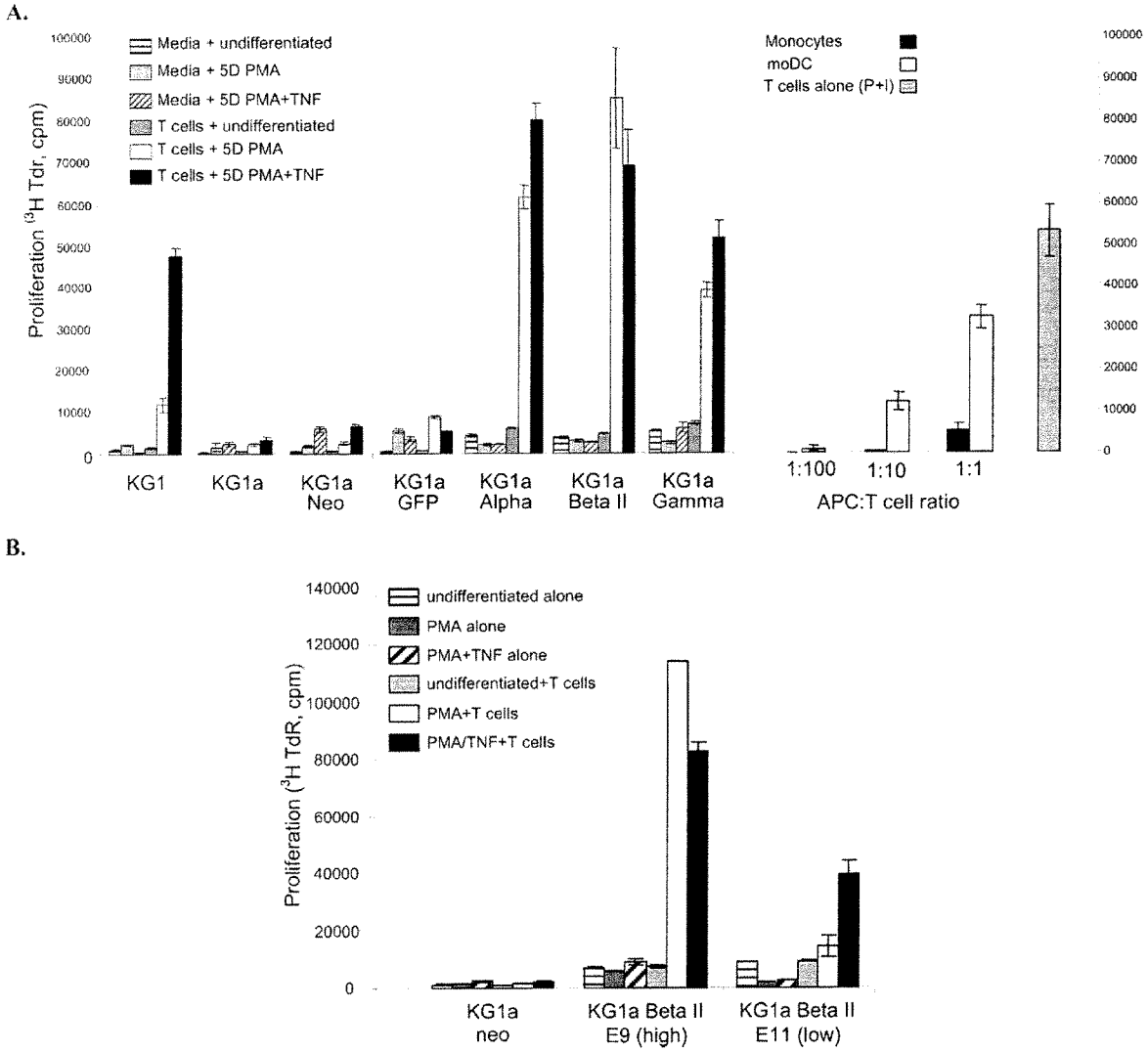

FIG. 7. **Induction T cell alloproliferation.** *A*, KG1, KG1a, and KG1a transfectants. KG1, KG1a, and KG1a transfectants were either left undifferentiated, or differentiated with PMA or PMA + TNF-α as indicated (*left panel*). These cells were then irradiated and cultured alone (*media*) or co-cultured at a 1:1 ratio with 2×10^5 purified normal T cells (*T cells*). Normal monocytes were either undifferentiated (*monocytes*) or differentiated with GM-CSF + IL-4 + TNF-α (*moDC*), irradiated, and co-cultured with the same donor T cells at the indicated ratios (*right panel*). T cells alone activated with PMA + ionomycin as a positive control. [^{3}H]Thymidine incorporation was measured for the last 18 h of culture. Data are presented as mean proliferation ± S.D. of triplicate wells. Results are representative of four independent experiments. *B*, high *versus* low PKC-βII-expressing KG1a clones. KG1a-neo, KG1a-βII-GFP high expresser (E9), or low expresser (E11) clones were left untreated or differentiated in PMA + TNF-α, irradiated, and co-cultured with the same normal donor T cells at a 1:1 ratio. [^{3}H]Thymidine incorporation was measured for the last 18 h of culture. Data are presented as mean proliferation ± S.D. of triplicate wells. Results are representative of two independent experiments.

PKC molecules. We first measured total PKC kinase activity under steady state conditions. As seen in Fig. 9*A*, unstimulated KG1 had more kinase activity than KG1a or KG1a-neo, whereas KG1a-βII (E9) had significantly more than both. To determine whether this increased PKC kinase activity resulted in increased PKC-β promoter activity, we cloned a 1.2-kb fragment of the human PKC promoter containing the previously reported PMA-inducible site (−11 to +43 (66)) into a luciferase reporter construct (pPKC-Forward). There was significantly higher luciferase activity when pPKC-Forward (but not the reverse orientation and empty vector negative controls) was transfected into E9 compared with the KG1a-neo control transfectants, demonstrating a 5-fold increase in promoter activity under unstimulated conditions (Fig. 9*B*). Finally, if an increase in constitutive PKC activity is inducing PKC-β expression, inhibition of this kinase activity would result in decreased expression. Consistent with this, unstimulated PKC-βII (E9) transfectants cultured with the PKC inhibitor bis I undergo a loss of both PKC-βII protein and mRNA expression (Fig. 9*C*). Together, these data indicate that constitutive PKC kinase activity can positively autoregulate PKC-β gene expression by PKC-responsive elements in the promoter.

DISCUSSION

Our findings support a model where quantitative and qualitative PKC-βII activation is a key component of the signal transduction pathways that induce DC differentiation and that regulation of this activation is in part through regulation of PKC-β

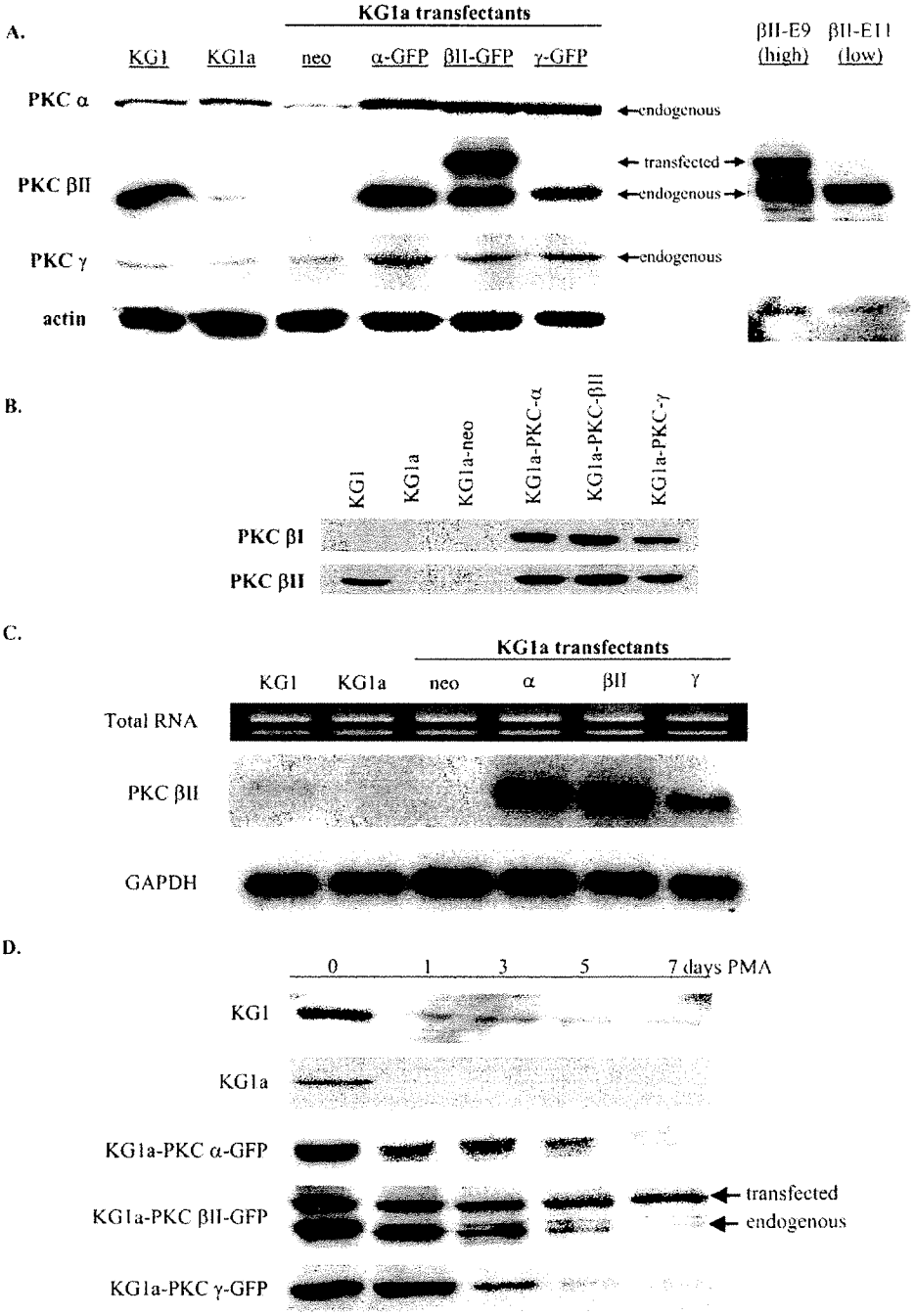

FIG. 8. **Up-regulation of endogenous PKC-βII expression.** *A*, induction of endogenous PKC-βII protein expression. Equal amounts of protein lysate from the unstimulated cells indicated were analyzed for PKC isoform expression by Western blot. *Arrows* point to the (larger) transfected PKC-GFP and (smaller) endogenous PKC bands. Blots were reprobed with anti-actin antibodies as a loading control. *B*, PKC-βI *versus* -βII expression. Protein lysates were analyzed as above using antibodies specific for PKC-βI or -βII. *C*, mRNA expression. Total mRNA was isolated from the indicated (unstimulated) cells, equalized by serial dilution (*top panel, Total RNA*), and analyzed by Northern blot for PKC-βII expression (using a PKC-βII-specific probe) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (as a loading control). *D*, degradation of PKC-βII following PMA activation. The indicated cells were cultured in PMA for the times indicated; protein lysates were made and equal amounts were analyzed for PKC-βII expression by Western blot. All results are representative of two independent experiments.

gene expression. Specifically, these data indicate the following. 1) PKC is a critical downstream signaling component of cytokine-induced monocyte→DC differentiation, consistent with our previous findings in $CD34^+$ HPC→DC differentiation (27). 2) Of the PKC isoforms, PKC-βII is the most centrally involved in DC differentiation. 3) Different aspects of DC differentiation are induced by differing degrees of PKC signal strength. 4) PKC signal strength is regulated (at least in part) by PKC-β gene expression,

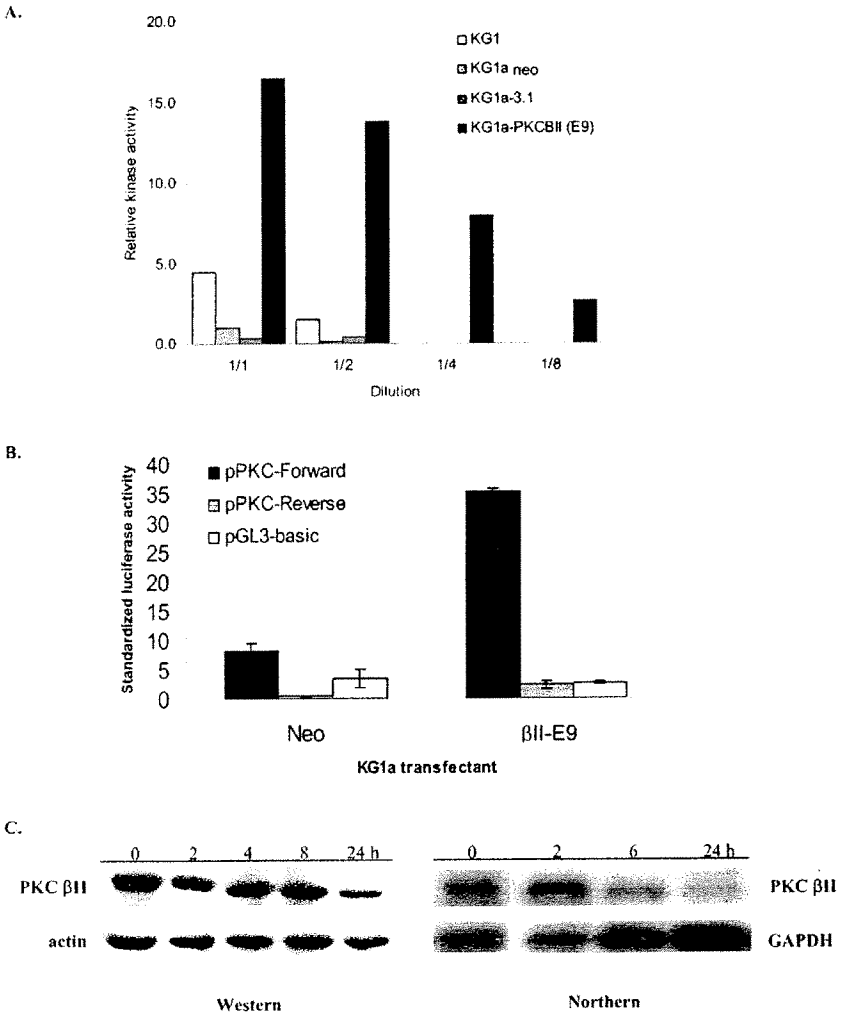

FIG. 9. **Regulation of endogenous PKC-βII by constitutive PKC kinase activity.** *A*, PKC kinase activity. Unstimulated KG1, KG1a, KG1a-neo, and KG1a-PKC-βII-GFP (clone E9) were analyzed for PKC kinase activity as detailed under "Materials and Methods." Activity was normalized to protein content and expressed relative to the activity of the undiluted (*1/1*) KG1a sample. *B*, PKC-β promoter activity. A 1.2-kb fragment of the PKC-β promoter was cloned into the forward (*pPKC-Forward*) or reverse (*pPKC-Reverse*) orientation into the pGL3-basic vector. Unstimulated KG1a-neo or KG1a-PKC-βII-GFP (clone E9) were transiently transfected with the indicated vectors (including the empty vector *pGL3-basic*), and luciferase activity was assessed as detailed under "Materials and Methods." *C*, effect of PKC inhibition. Unstimulated KG1a-PKC-βII-GFP (clone E9) cells were cultured with bisindolylmaleimide I for the times indicated, and PKC-βII expression was assessed by Western (*left*) and Northern (*right*) blot analysis. All the results shown are representative of two independent experiments. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

which involves a positive autoregulatory feedback loop.

The ability of the PKC inhibitor bisindolylmaleimide I to inhibit cytokine-driven monocyte (or $CD34^+$ HPC) progenitor to DC differentiation indicates that PKC is a downstream component of these signaling pathways. It is not yet clear which cytokine receptor is triggering PKC activation, as these receptors typically signal via Janus tyrosine kinase/signal transducers and activators of transcription and TRAFs, whereas PKC has been best characterized to be activated by G-coupled proteins and phospholipase C. However, the PKC adapter protein RACK-1 associates constitutively with the common β chain of the GM-CSF/IL-3/IL-5 receptor, and PKC-βII rapidly associates with this complex upon receptor activation (28).

Involvement of PKC-βII in GM-CSF receptor signaling is consistent with our observation that this isoform is activated in $CD34^+$ HPC by GM-CSF + TNF-α stimulation, and appears to be the cPKC isoform that is predominantly activated by PMA in three different DC progenitors. PKC-βII has also been implicated previously in myeloid differentiation (40), and our findings demonstrate a correlation between PKC-βII expression and the ability to undergo DC differentiation in KG1 and KG1a. The rapid activation of PKC-βII indicates that it is involved in the primary signaling rather than a later secondary response. Together, these data suggest that DC differentiation is specifically signaled through PKC-βII. However, we could not rule out the involvement of low level activation of the other cPKCs, activation of novel nPKC, and/or atypical PKC isoforms, and the formal possibility that PMA is acting through a non-kinase phorbol ester receptor (68) and that PKC-βII activation is an epiphenomenon.

To address these possibilities, we asked the seemingly straightforward question whether reconstitution of specific

PKC isoform expression would reconstitute the ability of KG1a to undergo DC differentiation, providing strong evidence for a central role for that isoenzyme. The inability of KG1a to undergo differentiation (57) has been attributed to lower expression of PKC isoforms/activity (37). Confocal studies of the KG1a-PKC-GFP transfectants demonstrated that only PKC-βII-GFP was clearly activated by PMA, consistent with what we saw for the endogenous cPKC. Most surprisingly, however, each of the PKC-GFP isoforms could reconstitute the ability of KG1a to undergo DC differentiation as assessed by inhibition of cell proliferation and induction of cell death, costimulatory ligand expression, re-establishment of inducible Rel B expression, and acquisition of allostimulatory capability. Although PKC-GFP transfection did not restore the high level MHC II surface expression seen on KG1, it did restore γ-interferon inducibility as seen by intracellular staining. These findings suggest that although some aspects of MHC II expression are regulated by PKC signaling, transport/processing for surface expression is not. The transfected KG1a also down-regulated constitutive MHC I expression, which is likely due to the increased constitutive PKC activity in these cells (and is consistent with the observation that PMA-induced PKC activation down-regulates MHC I (*e.g.* Fig. 4*C*)).

The unexpected the expression of CD40, CD80, CD86, and CD83 on the unstimulated KG1a-PKC-GFP transfectants is also likely due to increased constitutive PKC activity that is seen in the kinase assays. Most interestingly, this is the same "semi-activated" phenotype of the pathogenic Langerhans cells in Langerhans cell histiocytosis (69). It is unclear whether the variation in surface marker expression between the PKC-α, -βII, and -γ transfectants is due to qualitative and/or quantitative differences between the clones. However, the difference in MHC I, MHC II, CD40, and CD80 expression between the low (E11) and high (E9) expresser of PKC-βII suggests that the degree of PKC signal strength plays a defining role in determining which DC characteristics are manifested. This is supported by the observation that even though unstimulated KG1a-PKC-GFP expresses MHC and costimulatory ligands, they are still unable to activated T cells unless PKC is fully activated by an exogenous PKC agonist. Why there is no direct correlation between MHC/costimulatory ligand expression and the ability to activate T cells is unclear, although we and others have seen the same phenomenon in other myeloid DC progenitors (15, 70). Recently, Benvenuti *et al.* (60) have reported that even though DC from Rac $1/2^{-/-}$ mice have the same degree of MHC/costimulatory ligand expression as WT DC, they are far less capable of priming naive T cells. This appears to be in part due to the inability of these knockout DC to form the DC membrane extensions that physically interact with the T cell. Similarly, we believe that factors responsive to PKC signaling are involved in these initial physical/adhesive interactions and may be playing an important role in the immunogenicity of the PKC transfectants.

Varying degrees of PKC signaling is also supported by the confocal microscopy studies, where significant translocation of activated PKC-βII-GFP in the KG1a transfectants is only seen after PMA treatment. Further evidence for a differential effect of PKC signal strength is seen in the regulation of Rel B expression. These data and additional transcriptional run-off studies[2] suggest that the absence of PKC-βII activity (KG1a) results in constitutive/non-inducible Rel B expression; intermediate activity (unstimulated KG1 and KG1a-PKC-GFP) suppresses expression, and high PKC activity (PMA-stimulated KG1, KG1a-PKC-GFP) induces Rel B expression. Most interestingly, our EMSA studies suggest that the other NFκB families are similarly regulated. These data support a model where a continuous gradient of PKC activation elicits different aspects of DC differentiation and is similar to a model of differential lineage commitment based on the degree of PKC activation in other myeloid lineages (43). Although PKC signal strength has not been examined previously in DC differentiation, it has been shown that different degrees of CD40 signaling (which activates downstream PKC signaling in other cells (29, 30)) elicits different levels of DC migratory ability and cytokine secretion (71).

The tight association between PKC expression level and signaling activity is predicted by the steady state K_{act} relationship such that the increase in total PKC concentration in the KG1a transfectants results in an increase in the number of active PKC molecules. The ability of PKC expression levels to affect significantly PKC signaling is underscored by observations in a wide range of other cell types; the levels of mature enzyme are tightly regulated during development, and even modest changes in these levels can result in significant biological effects (66). It has also been suggested that other signaling pathways may work by regulating PKC expression, such as the ability of vitamin D_3 to drive HL-60 differentiation via up-regulation of PKC-β expression (72).

Although PKC protein expression and function has been shown to be tightly regulated by many post-transcription and post-translational mechanisms (65), regulation of PKC gene expression is largely uncharacterized. We have found that all three of the transfected cPKCs increase PKC expression, not through overexpression of the transfected cDNA (which is low for all the clones except βII-E9) but rather through significant induction of endogenous PKC-βII gene expression. Our promoter and PKC inhibitor studies suggest that the underlying mechanism involves a positive autoregulatory loop where increased constitutive PKC signaling activates a phorbol ester-inducible element in the PKC-β promoter, which has been reported to up-regulate gene expression 8–20-fold (66, 67, 73). We also see evidence that PKC-α and PKC-γ expression is similarly auto-induced (although to a much lesser extent than PKC-βII), and phorbol ester-inducible elements have also been described in the promoters of these genes (67, 74). Although not reported for hematopoietic cells, Liu *et al.* (44) have recently reported the same mechanism of autoregulation of steady state PKC-β gene expression in colonic epithelial cells, involving up-regulation of the PKC-β promoter by activated PKC and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-dependent signaling. Thus PKC autoregulation of its own expression may be a general characteristic of the cPKC isoforms in a variety of cell types.

Because of this interrelationship, however, we cannot definitively say that PKC-βII is the only cPKC involved in DC differentiation, given that endogenous α and γ are also up-regulated in the KG1a transfectants. However, there is reasonably strong evidence that PKC-βII is playing the major role. It is the only isoform that is consistently activated during DC differentiation in a number of different progenitors. It is only cPKC that has significantly different expression in KG1 *versus* KG1a. Differences in PKC-βII levels (*e.g.* E9 *versus* E11, which do not differ substantially in PKC-α or -γ expression) correlate with differences in DC surface markers. It would not, however, be surprising if there was some degree of functional redundancy between the cPKC isoforms.

Finally, the ability of KG1a to stably express endogenous PKC-βII through auto-induction raises the question how they lost this expression in the first place. One possibility is that the PKC-β promoter is also negatively regulated, and inhibitory

[2] Cejas, P. J. (2005) *Mol. Cell. Biol.*, in press.

elements have been described between −3000 and −690 of the 5′-untranslated region (which may not be included in our promoter construct) (67). If exogenous stimuli can inhibit PKC-β expression through these elements, this may provide a mechanism by which progenitor to DC differentiation can be inhibited in inflammatory settings where an adaptive immune response is unwanted (*e.g.* sterile trauma and wound healing). Although such a PKC-specific checkpoint has not been described, it has been well established that a number of cytokines (in particular those secreted by tumors) can inhibit the differentiation of myeloid progenitors to DC through signaling to yet undefined downstream targets (17).

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